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The effect of a 2-h exposure to cigarette smoke on the metabolic activation of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mice

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Abstract

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific nitrosamine, induces lung adenomas in A/J mice following a single intraperitoneal (i.p.) injection. However, inhalation of mainstream cigarette smoke does not induce or promote NNK-induced lung tumors in this mouse strain purported to be sensitive to chemically-induced lung tumorigenesis. The critical events for NNK-induced lung tumorigenesis in A/J mice is thought to involve O^6-methylguanine (O^6MeG) adduct formation, GC→AT transitional mispairing, and activation of the K-*ras* proto-oncogene. The objective of this study was to test the hypothesis that a smoke-induced shift in NNK metabolism led to the observed decrease in O^6MeG adducts in the lung and liver of A/J mice co-administered NNK with a concomitant 2-h exposure to cigarette smoke as observed in previous studies. Following 2 h nose-only exposure to mainstream cigarette smoke (600 mg total suspended particulates/m^3 of air), mice ($n = 12$) were administered 7.5 μmol NNK (10 μCi [5-^{3}H]NNK) by i.p. injection. A control group of 12 mice was sham-exposed to HEPA-filtered air for 2 h prior to i.p. administration of 7.5 μmol NNK (10 μCi [5-^{3}H]NNK). Exposure to mainstream cigarette smoke had no effect on total excretion of NNK metabolites in 24 h urine; however, the

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metabolite pattern was significantly changed. Mice exposed to mainstream cigarette smoke excreted 25% more 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) than control mice, a statistically significant increase ($P < 0.0001$). Cigarette smoke exposure significantly reduced α-hydroxylation of NNK to potential methylating species; this is based on the 15% reduction in excretion of the 4-(3-pyridyl)-4-hydroxybutanoic acid and 42% reduction in excretion of 4-(3-pyridyl)-4-oxobutanoic acid versus control. Detoxication of NNK and NNAL by pyridine-*N*-oxidation, and glucuronidation of NNAL were not significantly different in the two groups of mice. The observed reduction in α-hydroxylation of NNK to potential methylating species in mainstream cigarette smoke-exposed A/J mice provides further mechanistic support for earlier studies demonstrating that concurrent inhalation of mainstream cigarette smoke results in a significant reduction of NNK-induced O^6MeG adduct formation in lung and liver of A/J mice compared to mice treated only with NNK.
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Keywords: Cigarette smoke; Tobacco-specific nitrosamine

Nomenclature

diol	4-(3-pyridyl)butane-1,4-diol
ETS	environmental tobacco smoke
hydroxy acid	4-(3-pyridyl)-4-hydroxybutanoic acid
keto alcohol	4-hydroxy-1-(3-pyridyl)-1-butanone
keto acid	4-(3-pyridyl)-4-oxobutanoic acid
7-MeG	7-methylguanine
NAT	*N*-nitrosoanatabine
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNAL-Gluc	[4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid
NNAL-*N*-oxide	4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNK-*N*-oxide	4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone
NNN	*N*-nitrosonornicotine
O^6MeG	O^6-methylguanine
O^4MeT	O^4-methylthymine
TSP	total suspended particulate
TSNA	tobacco-specific nitrosamine

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1. Introduction

It has been proposed that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)[1], a tobacco-specific nitrosamine (TSNA), may be involved in the causation of human lung cancer [1]. This hypothesis is supported by evidence showing that the lung is the main target tissue for NNK-induced tumorigenicity in laboratory animals, although direct evidence for the involvement of NNK in human lung cancer is lacking [2].

The metabolism of NNK is illustrated in Fig. 1 [2]. α-Hydroxylation of the methylene carbon atoms adjacent to the *N*-nitroso group is believed to be the major pathway of metabolic activation of NNK and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) since the unstable intermediates spontaneously decompose to 4-(3-pyridyl)-4-oxobutanoic acid (keto acid) and 4-(3-pyridyl)-4-hydroxybutanoic acid (hydroxy acid), respectively. Decomposition of the reactive intermediates in both pathways has the potential to induce methylation of DNA. In contrast, α-hydroxylation of the NNK methyl group yields an unstable intermediate which spontaneously decomposes to 4-hydroxy-1-(3-pyridyl)-1-butanone (keto alcohol; also referred to as HPB) or HPB-releasing adduct(s). α-Methyl hydroxylation of NNAL produces a similar cascade of products, the major end product of which is 4-(3-pyridyl)butane-1,4-diol. However, this latter pathway is not known to result in pyridylhydroxybutylation of DNA. Detoxification of NNK occurs by glucuronidation of NNAL to yield [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glu-

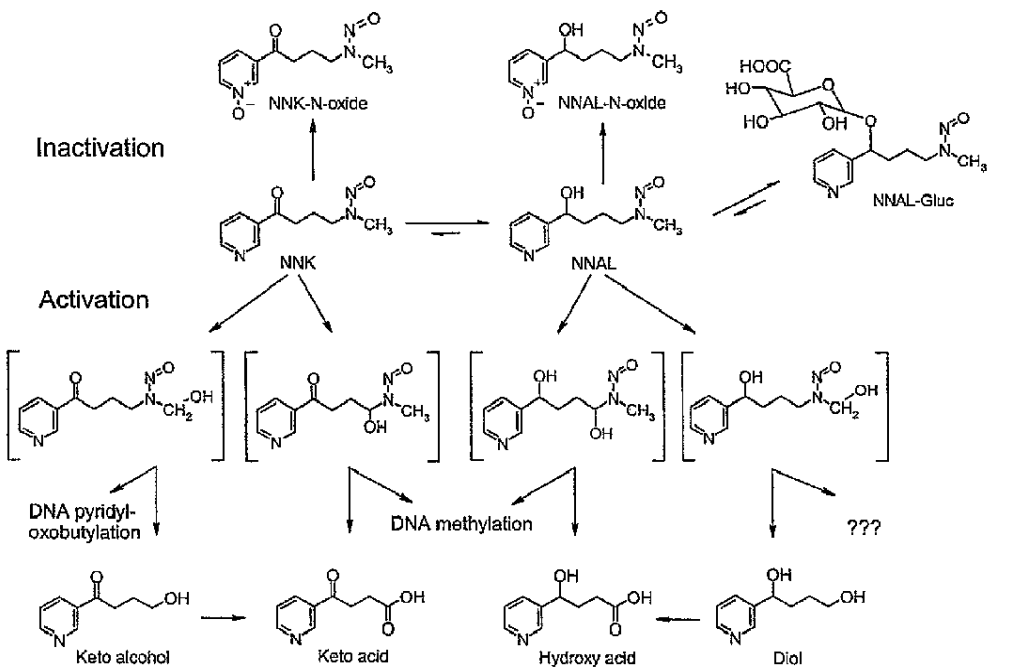

Fig. 1. NNK metabolism pathways based on studies in laboratory animals.

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cosiduronic acid (NNAL-Gluc), and by pyridine-*N*-oxidation of NNK and NNAL to yield 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone (NNK-*N*-oxide) and 4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-butanol (NNAL-*N*-oxide), respectively.

In the A/J mouse lung tumor model, a single intraperitoneal (i.p.) injection of 10 μmol [2.07 mg] NNK/mouse results in 7–12 lung tumors per mouse after 16 weeks [3]. The lowest dose of NNK to induce a statistically significant increase in lung tumor multiplicity corresponds to a single i.p. injection of 2.5 μmol NNK/mouse [4]. The initial event in NNK-induced lung tumorigenesis in the A/J mouse is believed to be the formation of O^6-methylguanine (O^6MeG), a major promutagenic adduct that leads to GC→AT transitional mispairing and subsequent activation of the K-*ras* proto-oncogene [4,5]. Although NNK readily induces lung tumors in A/J mice, which are highly sensitive to chemically-induced lung tumorigenesis compared to more resistant mouse strains [6], inhalation of mainstream cigarette smoke [7] and sidestream cigarette smoke [8] fails to induce lung tumors in A/J mice. Furthermore, exposure of A/J mice to mainstream cigarette smoke does not promote NNK-induced tumors [7]. A recent study has reported a small yet statistically significant tumorigenic response in the A/J mouse using gas phase only of tobacco smoke at high doses; however, the authors concluded that TSNA were not the causative agent in the A/J mouse because the amount of TSNA in the smoke was orders of magnitude below what is required to induce even one tumor in the A/J mouse via systemic injection [9].

Since our previous study demonstrated that nose-only inhalation exposure of A/J mice to mainstream cigarette smoke results in a significant dose-dependent reduction in NNK-induced lung and liver O^6MeG adduct formation [10], the objective of the current study was to test the hypothesis that a 2 h exposure to tobacco smoke results in a shift in NNK metabolism that led to the observed decrease in NNK-induced DNA adducts.

2. Materials and methods

2.1. Animals

Female A/J mice (4–5 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were housed individually in polycarbonate cages bedded with Alpha-Dri™ (Shepherd Specialty Papers, Inc.; Kalamazoo, MI). Animals had ad libitum access to water and pelleted AIN-76A diet (Dyets Inc.; Bethlehem, PA) except during inhalation of tobacco smoke. The animals were housed in a vivarium with a day–night cycle of 12 h (darkness beginning at 18:00), temperature (18–26 °C), relative humidity (30–70%), and airflow with a minimum of ten air changes per hour. Experimental protocols involving the use of animals must be reviewed and approved by the R.J. Reynolds Tobacco Company Institutional Animal Care and Use Committee.

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2.2. Materials

Kentucky 1R4F reference research cigarettes were obtained from the Tobacco Research Institute (University of Kentucky; Lexington, KY) and stored at 4 °C until use. [5-^{3}H]NNK, with a specific activity of 5 Ci/mmol, and NNK were purchased from ChemSyn Laboratories (Lenexa, KS). NNK metabolites were a generous gift from Dr S. Amin (American Health Foundation; Valhalla, NY). All other chemicals were either of HPLC or analytical grade (Merck; Darmstadt, Germany).

2.3. Nose-only smoke exposure

Following acclimatization for 14 days, animals were randomly assigned to two groups based on body weight to insure a balanced variance during study conduct. Control animals ($n = 12$) were sham-exposed by 2 h nose-only inhalation to HEPA-filtered air and experimental ($n = 12$) animals were exposed by 2 h nose-only inhalation to mainstream cigarette smoke from the Kentucky 1R4F reference research cigarette. The exposure conditions have been previously described by Brown et al. [10]. Briefly, cigarettes were conditioned for 24–72 h in a Climate-Lab® cabinet (PGC; Black Mountain, NC) at 40–70% relative humidity and 18–26 °C prior to smoking. Mainstream smoke was generated using a 30-port, computer-controlled smoke generator [11] under Federal Trade Commission (FTC) smoking conditions of a 2 s-puff of 35 ml volume, and a frequency of one puff per min. Smoke was directed into a mixing flask, and diluted with HEPA-filtered and humidified air prior to entering the nose-only exposure chamber. The exposure atmosphere had a relative humidity of 40–70% and a temperature of 18–26 °C. Breathing zone smoke concentrations were monitored by a RAM-1 aerosol concentration analyser (MEI Corp.; Billerica, MA), and the concentration of smoke in the nose-only exposure chamber was controlled by a feedback-loop exposure controller. The mainstream smoke exposure concentration used in the study was 600 mg total suspended particulate (TSP)/m^3.

2.4. NNK administration

Immediately following a 2 h-exposure to either HEPA-filtered air or mainstream cigarette smoke, animals were administered a single i.p. dose of 7.5 μmol NNK (10 μCi [5-^{3}H]NNK) in physiological saline. Animals were then placed in individual metabolism cages. [5-^{3}H]NNK was administered post-smoke exposure to prevent contamination of the smoke machines and laboratory personnel from [5-^{3}H]NNK that could possibly be in the excreted urine of the animals during smoke exposure.

2.5. NNK exposure from cigarette smoke

A Kentucky 1R4F reference cigarette contains ≈84 ng NNK. Based on a 10.8 mg WTPM yield from the cigarette and with an exposure concentration of cigarette

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smoke to be 0.6 mg WTPM/l of air, NNK is estimated to be present at a concentration of 4.67 ng/l in the exposure smoke. Assuming that each mouse inhaled only 8 ml/min for 120 min, the amount of inhaled NNK from cigarette smoke exposure would be 4.48 ng.

2.6. Collection of urine

Urine was collected over a 24 h time interval in polyethylene vials kept cold with ice. After the 24 h collection period, animals were removed from the cages, and the cages washed with distilled water to collect as much urine as possible, i.e., including urine that may have attached to the walls of the cages. Final urine samples were brought to a total volume of 15 ml using distilled water. Urine samples were stored at -20 °C until such time that HPLC analysis was performed.

2.7. HPLC analysis

Urine samples were centrifuged at $10000 \times g$ to remove solid debris, then the supernatant freeze dried and reconstituted in distilled water. Aliquots (0.2–0.5 ml) were chromatographed on a 4.6×250 mm^2 LiChrosorb® RP18 SelectB column (Merck) [12]. Gradient program conditions were 100% of solvent A for 3 min, linear to 70% solvent A/30% solvent B in 37 min, linear to 50% solvent A/50% solvent B in 2 min, and linear back to 100% solvent A within 2 min (solvent A: 20 mM phosphate buffer, pH 6.0; solvent B: acetonitrile) at a flow rate of 0.7 ml/min. Tritium was detected by solid-phase radioactivity monitoring (Ramona, Raytest; Straubenhardt, Germany). Radioactive metabolites were identified by co-chromatography with unlabeled reference compounds using UV detection at 233 nm. The identity of NNAL-Gluc was confirmed by co-chromatography with [1-^{14}C]NNAL-Gluc obtained from a previous study [13], and by treatment of urine aliquots with β-glucuronidase [14]. The distribution of radioactive metabolites was determined by integration of the individual peaks and calculation of the percentage of total activity eluting from the column.

2.8. Statistical analysis

Reported values represent means ± standard error (SE). Statistical analysis was performed by the two-tailed t-test for independent samples.

3. Results

The urinary excretion of NNK was studied in A/J mice administered a single i.p. dose of 7.5 μmol NNK after 2 h nose-only inhalation exposure to either HEPA-filtered air or 600 mg TPM/m^3 mainstream smoke from Kentucky 1R4F reference research cigarettes. Between 45 and 85% of total urinary excretion of NNK occurred within the first 24 h, with no significant difference between sham-exposed ($67.4 \pm 2.8\%$) and smoke-exposed animals ($64.5 \pm 2.8\%$).

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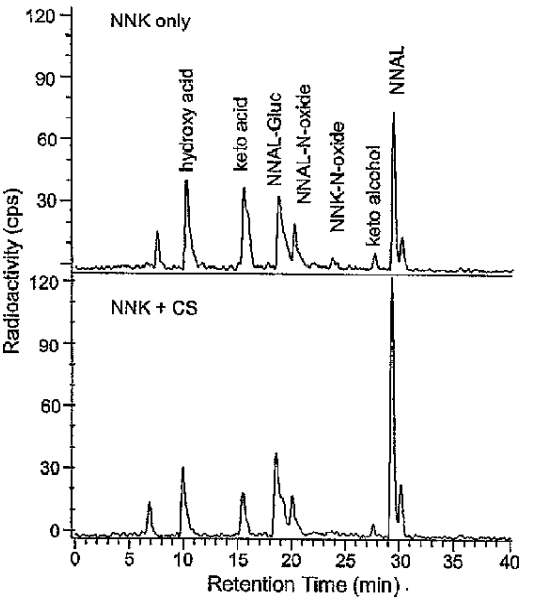

Fig. 2. HPLC analysis of urinary metabolites from A/J mice 24 h post i.p. administration of [5-^{3}H]NNK (7.5 μmol/mouse) following 2 h nose-only exposure to HEPA-filtered air (upper trace) or mainstream cigarette smoke (CS; lower trace).

Fig. 2 shows a typical HPLC profile of NNK metabolites in 24 h urine while Fig. 3 shows the pattern of major NNK metabolites detected in 24 h urine. Cigarette smoke exposure significantly increased ($P < 0.0001$) excretion of NNAL by 25% compared to control mice. Cigarette smoke exposure significantly reduced the excretion of α-hydroxylation metabolites hydroxy acid and keto acid by 15% ($P = 0.0029$) and 42% ($P < 0.0001$) versus NNK-treated control mice, respectively. Excretion of the detoxication products NNAL-Gluc and NNAL-*N*-oxide was not significantly different between the two groups of mice. Only three of the 12 mice exposed to NNK only excreted NNK-*N*-oxide compared to none of the NNK plus cigarette smoke exposure group. The difference was not significant.

4. Discussion

Aqueous solutions of cigarette smoke particulate matter, nicotine, and cotinine significantly reduce the genotoxicity of NNK in both Ames mutagenicity and sister chromatid exchange assays [15]. Tobacco smoke and tobacco smoke constituents also modify both the in vitro and in vivo metabolism of NNK in laboratory animals [2]. Nicotine and the TSNA *N*-nitrosonornicotine and *N*-nitrosoanatabine (NAT) show dose-dependent inhibition of in vitro α-hydroxylation of NNK by rat oral tissue [16]. Nicotine and cotinine both reduce NNK metabolic activation by α-hydroxylation in the isolated and perfused rat liver, but not in the isolated and

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perfused rat lung [17]. Nicotine significantly reduces in vivo metabolic activation of NNK and excretion of α-hydroxylation metabolites [12], and significantly reduces [5-^{3}H]NNK binding of radioactivity (pyridyloxobutylation) to rat hemoglobin [18].

Nicotine inhibits in vitro α-hydroxylation of NNK and protein binding (pyridyloxobutylation) in hamster lung explants [19] and hepatic microsomal proteins [20]. Compared to other rodent species, the hamster is not very sensitive to NNK-induced lung tumorigenesis [2], probably as a consequence of limited NNK α-hydroxylation in the lung [21]. Exposure of hamsters to mainstream smoke does not increase the incidence of respiratory tract tumors following administration of NNK [22].

In the A/J mouse, inhalation exposure to mainstream cigarette smoke [7] and sidestream cigarette smoke [8] fails to induce a tumorigenic response in the lung. However, a recent study has reported that whole-body inhalation exposure of A/J mice to 11% mainstream cigarette smoke and 89% sidestream cigarette smoke, used as an experimental surrogate for environmental tobacco smoke (ETS), results in a tumorigenic response [9]. Mice were exposed to the ETS surrogate at a concentration of 87.3 ± 21.0 mg TSP/m^3, 244 ± 40 ppm CO, and 16.1 ± 6.3 mg nicotine/m^3

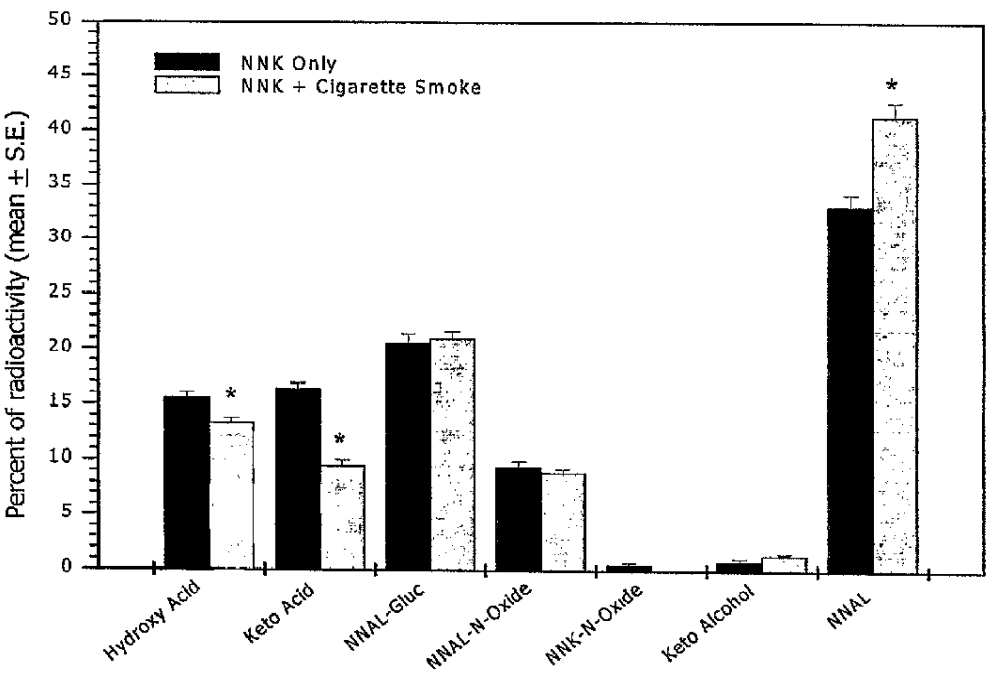

Fig. 3. Effect of 2 h exposure to mainstream cigarette smoke (600 mg TSP/m^3) on the metabolite pattern in A/J mice 24 h urine post i.p. administration of [5-^{3}H]NNK (7.5 μmol/mouse). Sum of alpha includes hydroxy acid, keto acid, and keto alcohol. NNAL metabolites include hydroxy acid, NNAL-Gluc, NNAL-*N*-oxide, and NNAL. NNK metabolites include keto acid, NNK-*N*-oxide, and keto alcohol. Values are the mean ± SE of 12 animals. Asterisks indicate a statistically significant difference from control mice only at $P < 0.01$.

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for 6 h/day, 5 days/week for 5 months, followed by a 4 month post-exposure recovery period in air. In a second study, A/J mice were exposed to the same surrogate for whole ETS (78.5 ± 12.4 mg TSP/m^3, 211 ± 24 ppm CO and 13.4 ± 3.3 mg nicotine/m^3) or to HEPA-filtered gas phase of the ETS surrogate (0.1 ± 0.2 mg TSP/m^3, 113 ± 23 ppm CO and 3.1 ± 2.0 mg nicotine/m^3) for 6 h/day, 5 days/week for 5 months followed by a recovery period of 4 months in air [23]. Both exposure regimens resulted in a non-significant increase in tumor incidence and a significant increase in lung tumor multiplicity. The concentration of NNK (mean ± S.D.) in the whole ETS surrogate and gas-phase ETS surrogate exposure atmospheres was 3.9 ± 3.5 and 0.29 ± 0.28 μg/m^3, respectively; based on these values, the authors concluded that the maximum uptake of NNK by mice during the exposure periods was at least four orders of magnitude lower than the predicted unit dose required to induce a one-tumor response in A/J mice. While the above studies suggest that certain experimental exposure protocols to tobacco smoke may result in a tumorigenic response in the A/J mouse lung [9,23], the relevance of these high experimental exposure conditions to an ETS surrogate are unclear; this, given that typical personal exposure concentrations among non-smokers exposed to particulate matter associated with ETS has been estimated to range from 18 to 64 μg/m^3 [24].

The relevance of NNK to the induction of lung tumors in cigarette smoke-exposed A/J mice [22,23] is further questioned in mechanistic studies investigating the biological activity of NNK in the presence of tobacco smoke [10]. Exposure of A/J mice to mainstream cigarette smoke (0, 400, 600, or 800 mg TSP/m^3) does not result in detectable levels of O^{6}MeG in either lung or liver [10]. Moreover, A/J mice co-exposed to mainstream cigarette smoke (0, 400, 600, or 800 mg TSP/m^3) and a single i.p. administration of NNK (0, 3.75, or 7.5 μmol/mouse), exposure to tobacco smoke resulted in a significant dose-dependent reduction in NNK-induced lung and liver O^{6}MeG [10]. The current study in which A/J mice were exposed to mainstream cigarette smoke from the 1R4F cigarette (600 mg TSP/m^3) for 2 h followed by a single i.p. injection of NNK (7.5 μmol/mouse), shows that tobacco smoke exposure results in a significant reduction in NNK metabolic activation to hydroxy acid and keto acid by 15% ($P = 0.0029$) and 42% ($P < 0.0001$) versus NNK-treated control mice, respectively. In the absence of additional data on the chronic effect of tobacco smoke exposure on enzyme induction and inhibition, it can only be concluded that a 2-h co-administration of tobacco smoke appears to reduce the metabolic activation of NNK by α-hydroxylation to DNA-reactive methylating species, a critical step in the induction of lung tumorigenesis in the A/J mouse. Longer-term exposures to tobacco smoke and concomitant exposure to high doses of NNK have not been reported in the literature. Under these longer-term conditions, results could be different than the observed results of the 2-h exposure that is reported here.

While this study provides no explanation for the observed increase in lung tumorigenesis in A/J mice following exposure to tobacco smoke under certain experimental conditions [9,23], the current results, and those from our previous study [10], support the hypothesis that a 2 h tobacco smoke exposure results in a shift in NNK metabolism that could lead to the observed decrease in NNK-induced DNA adducts.

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